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3-Acyl dihydroflavonols from poplar resins collected by honey bees are active against the bee pathogens *Paenibacillus larvae* and *Ascosphaera apis*

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ABSTRACT

Honey bees, *Apis mellifera*, collect antimicrobial plant resins from the environment and deposit them in their nests as propolis. This behavior is of practical concern to beekeepers since the presence of propolis in the hive has a variety of benefits, including the suppression of disease symptoms. To connect the benefits that bees derive from propolis with particular resinous plants, we determined the identity and botanical origin of propolis compounds active against bee pathogens using bioassay-guided fractionation against the bacterium *Paenibacillus larvae*, the causative agent of American foulbrood. Eleven dihydroflavonols were isolated from propolis collected in Fallon, NV, including pinobanksin-3-octanoate. This hitherto unknown derivative and five other 3-acyl-dihydroflavonols showed inhibitory activity against both *P. larvae* (IC_{50} = 17–68 μM) and *Ascosphaera apis* (IC_{50} = 8–23 μM), the fungal agent of chalkbrood. A structure-activity relationship between acyl group size and antimicrobial activity was found, with longer acyl groups increasing activity against *P. larvae* and shorter acyl groups increasing activity against *A. apis*. Finally, it was determined that the isolated 3-acyl-dihydroflavonols originated from *Populus fremontii*, and further analysis showed these compounds can also be found in other North American *Populus* spp.

1. Introduction

'Propolis' is the apicultural term for a sticky nest-building material that bees make by mixing foraged plant resins with wax. Propolis deposition in managed hives of honey bees has been considered a nuisance and consequently selected against in bee breeding; however, it is now well established that this behavior is an important adaptation for colony health. Natural or man-made propolis envelopes prevent the chronic up-regulation of individual immune function in managed hives (Borba et al., 2015; Simone et al., 2009), which is a trade-off with colony productivity (Evans and Pettis, 2005). In addition, honey bees that deposit more propolis in the hive tend to live longer and have healthier brood (Nicodemo et al., 2014). This relationship between propolis and bee health make resin collection and propolis production important aspects of bee biology for beekeepers. Nevertheless, little is known about the compounds that make propolis useful to bees or from what plants they originate.

The antimicrobial activity of propolis is thought to be the basis of its benefit to bees. Propolis can inhibit the growth of several bee pathogens *in vitro* including the bacterium *Paenibacillus larvae*, the cause of American foulbrood, and the fungus *Ascospherea apis*, the cause of chalkbrood (Lindenfelser, 1967; Bastos et al., 2008; Wilson et al., 2015). IC_{50} values ranging from 41 to 120 μg/ml against *P. larvae* and 7–48 μg/ml against *A. apis* for propolis sampled from 12 different regions in the U.S. were observed in previous work (Wilson et al., 2015). In addition, the enrichment of managed

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colonies with propolis to simulate natural nesting conditions can prevent the clinical symptoms of American foulbrood and chalkbrood when colonies are challenged with *P. larvae* or *A. apis*, respectively (Simone-Finstrom and Spivak, 2012; Borba, 2015). Similarly, the presence of conifer resins in the nests of wood ants, another Hymenopteran insect, increases the survival of both adults and larvae when challenged with entopathogenic bacteria and fungi (Chapuisat et al., 2007).

The overall antimicrobial activity of propolis is a consequence of the different resin chemistries available to bees in different regions, and bees are known to collect resins from many plant species overall (Crane, 1990; Bankova, 2006; Wilson et al., 2013). Both honey bees and stingless bees can discriminate among multiple resinous plants (Leonhardt and Blüthgen, 2009; Wilson et al., 2013; Drescher et al., 2014), presumably making choices based on resin composition to maximize their benefit to the hive. The chemical composition of plant resins can be remarkably different, which has led to a diversity of antimicrobial activities found in various propolis studies (Kujumgiev et al., 1999; Bastos et al., 2008; Wilson et al., 2015). This diversity is illustrated by Lindenfelser in a comprehensive screen of 15 different U.S. propolis samples against 45 human, animal, and plant pathogens. Each individual pathogen was inhibited by at least one propolis sample at 100 μg/ml, but only *P. larvae* was inhibited by all 15 samples (Lindenfelser, 1967). Since bees often choose among many different resinous plants, this result raises the hypothesis that bees have evolved to collect resins that are specifically effective against their own suite of pathogens.

The antimicrobial compounds in propolis from temperate regions of Europe and the U.S. are thought to be flavonoids and/or organic acid esters originating mostly from poplar trees (*Populus* spp.) (Bankova, 2005, 2006), which secrete resin from young leaves and buds (Langenheim, 2003). Several known compounds with activity against *P. larvae* were previously isolated from Bulgarian propolis including pinocembrin, pinobanksin-3-acetate, and a mixture of caffeic acid esters (Bilikova et al., 2012). Minimum inhibitory concentrations (MIC) were reported as 31 μg/ml against enterobacterial repetitive intergenic consensus (ERIC) type I *P. larvae* for all compounds, with MIC = 62 μg/ml for pinocembrin and pinobanksin-3-acetate (**4**, Table 1) and MIC = 31 μg/ml for the caffeate mixture against ERIC type II *P. larvae* (Bilikova et al., 2012). However, these compounds were not likely responsible for the majority of anti-*P. larvae* activity previously observed in U.S. propolis samples, as anti-*P. larvae* activity was not correlated with the relative amounts of these compounds present among the samples studied (Wilson et al., 2015). Cinnamic acid and pinocembrin are two propolis components with known activity against *A. apis* (Voight and Rademacher, 2015).

North American poplar resins are chemically distinct and differentially inhibit the growth of *P. larvae* (Wilson et al., 2013), which raises questions about the usefulness of different poplar resins to bees. This differential activity may mean that poplar resins are not interchangeable without consequences to bee health; however, resins with less activity against *P. larvae* may contribute beneficial antimicrobial activity against other bee pathogens. Thus, the benefits of propolis may be maximized when diverse resin sources are available in the environment compared to the availability of a single resin with high specific activity. Knowing the identity and distribution of specific resin compounds beneficial to bees would provide a link between resin compounds and bee health that could be exploited in bee management.

Since the most sustainable solutions to bee decline will be derived from leveraging how bees naturally resist disease, discovering the identity, efficacy, and distribution of resin specialized metabolites that inhibit bee pathogens will enhance our ability to take advantage of the practical effects of plant resins on bee health. The goal of the present work was to use bioassay-guided fractionation and chemical analysis to determine the identity and botanical distribution of resin compounds active against *P. larvae* and *A. apis*. This work provides a basic link between specific products of plant metabolism and the benefits that bees derive from particular resins. This information could allow beekeepers to rationally modify the landscape to provide bees with resinous plants that maximize the health benefits of propolis, and thus maximize colony health. Propolis in the U.S. may also have significant untapped potential as a source of useful antimicrobial compounds, and discovering these compounds will potentially benefit beekeepers by adding commercial value to what is considered an annoying secondary hive product.

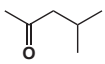
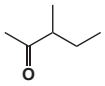
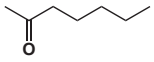
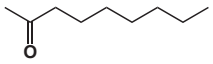
2. Results and discussion

2.1. Identification of antimicrobial compounds

An extract of propolis from Fallon, NV, with relatively high activity against *P. larvae* (IC_{50} = 41 μg/ml) and *A. apis* (IC_{50} = 8 μg/ml) among U.S. propolis samples in a previous study (Wilson et al., 2015) was subjected to bioassay-guided fractionation against *P. larvae*. 80 g powdered propolis was extracted with 70% aqueous ethanol and subsequently partitioned against hexanes and dichloromethane (CH_2Cl_2). The active CH_2Cl_2 fraction was further purified by normal-phase flash chromatography on silica, open column chromatography on Sephadex LH-20, and reversed-phase preparative HPLC on C_{18} (S1). This process yielded 10 known compounds (**1**–**10**) and one unknown compound (**11**) (Table 1). Isolation details and yields for compounds **1**–**10** and **11** can be found in S1 or the Experimental section, respectively.

All ^{1}H-NMR spectra showed characteristic flavonoid patterns (Fig. 1) (Markham, 1982). All compounds contained unsubstituted B-rings demonstrated by two multiplets at 7.5 and 7.4 ppm representing protons at positions 2′ and 6′ and protons at positions 3′-5′, respectively (Table 2) (Mabry et al., 1970; Markham, 1982). Two doublets at 5.96 ppm and 5.94 ppm representing coupled protons at positions 8 and 6 (^4J = 2 Hz) demonstrated that all compounds contained di-substituted A-rings (Mabry et al., 1970; Markham, 1982), while two more doublets at ~5.4 ppm and ~5.85 ppm representing coupled protons at positions 2 and 3 (^3J = 12 Hz) demonstrated that all compounds were dihydroflavonols (Table 2) (Mabry et al., 1970; Markham, 1982). The 12 Hz ^3J coupling between protons at positions 2 and 3 established these protons as *trans*-oriented in all isolated compounds (Table 2). Comparing the circular dichrosim (CD) spectra from the literature (Slade et al., 2005) with CD spectra for compounds **1**–**11** (Table 1) confirmed that positions 2 and 3 of all isolated compounds were in the naturally abundant 2R, 3R configuration with positive Δε values for the ~335 nm absorption band and negative Δε values for the ~295 nm absorption band (S4-S8). The absolute configuration of **10** was not confirmed due to limited amount of sample. The UV–Vis and LC-MS-MS spectra further supported that all isolated compounds were dihydroflavanols based on their universal absorption at ~290 and ~330 nm (S3) (Markham, 1982) and production of characteristic A-ring fragments (Table 3) (Pinheiro and Justino, 2012). LC-MS-MS data also showed distinct losses suggesting the presence of various acyl groups in compounds **4**–**11** (Table 3). Compounds **1**, **2**, and **4** were confirmed as pinobanksin (**1**), pinostrobin (**2**), pinobanksin-3-acetate (**4**) and by ^{1}H-NMR and LC-MS-MS comparison to authentic standards. These compounds have been previously isolated from European propolis (Marcucci, 1995). UV shift analysis with sodium acetate and aluminum chloride according to Markham (1982) confirmed that compound **3** was 5-O-methylpinobanksin (**3**), which has been previously isolated and characterized by NMR

Table 1
Chemical structures and activities of isolated compounds. Compounds that were less active than crude propolis extract in preliminary screens against *P. larvae* are denoted with (—). Compounds **6**–**7** and **8**–**9** were tested as mixtures and their IC_{50} values are denoted with (*).

Compound	R_1	R_2	R_3	*P. larvae* IC_{50} (μM)	*A. apis* IC_{50} (μM)
Pinobanksin (**1**)	OH	OH	H	—	—
Pinostrobin (**2**)	OCH_3	OH	H	—	—
5-O-methylpinobanksin (**3**)	OH	OCH_3	H	—	—
Pinobanksin-3-acetate (**4**)	OH	OH		—	—
Pinobanksin-3-propanoate (**5**)	OH	OH		—	—
Pinobanksin-3-butyrate (**6**)	OH	OH		*68 ± 17	*7.8 ± 0.5
Pinobanksin-3-isobutyrate (**7**)	OH	OH			
Pinobanksin-3-isopentanoate (**8**)	OH	OH		*39 ± 4	*8.3 ± 0.5
Pinobanksin-3-(2-methyl) butyrate (**9**)	OH	OH			
Pinobanksin-3-hexanoate (**10**)	OH	OH		22 ± 5	23 ± 2
Pinobanksin-3-octanoate (**11**)	OH	OH		17 ± 4	>250

from Bulgarian propolis (Bankova et al., 1983).

Comparison of spectra to a pinobanksin-3-acetate (**4**) standard indicated that the remaining unknowns were various 3-acyl-pinobanksin derivatives, which have been previously reported in propolis samples from Europe and Uruguay (Marcucci, 1995; Kumazawa et al., 2002; Falcão et al., 2010), and spectroscopic data for compounds **5**–**11** can be found in the supplementary data (S4-S8). The attachment point and branching of acyl groups has been ambiguous in studies relying on MS without absolute standards (e.g. Falcão et al., 2010); however, the attachment of acyl groups at the 3 position of the pinobanksin backbone was confirmed with the observation of ^3J coupling between the proton at position 3 and the carbonyl at position 1″ in heteronuclear multiple bond correlation (HMBC) experiments (Fig. 2, Fig. S4-S8), while correlation spectroscopy (COSY) experiments were used to determine acyl group branching (Fig. 2, Fig. S2). Compound **5** ([M-H] = 327.0872 *m/z*) displayed a fragmentation pattern and NMR data consistent with a three-carbon acyl group (Table 3, Fig. S2). These data are consistent with **5** being identified as pinobanksin-3-propanoate (**5**) (Table 1). Pinobanksin-3-propanoate (**5**) has been provisionally identified by MS alone in propolis from Europe (Falcão et al., 2010; Marcucci, 1995), Canada (García-Viguera et al., 1993), Iran (Mohammadzadeh et al., 2007), and Uruguay (Kumazawa et al., 2002).

The isomeric pair **6**–**7** ([M-H] = 341.1072 *m/z*) and **8**–**9** ([M-H] = 355.1182 *m/z*) could not be separated on the preparative scale by C_{18}, C_8, or NH_2 reversed-phase chromatography; however, each pair could be resolved enough on the analytical scale to produce pure MS spectra. Compounds **6**–**7** and **8**–**9** produced identical MS-MS fragmentation patterns, respectively, that were consistent with the presence of four-carbon acyl groups in **6**–**7** and five-carbon acyl groups in **8**–**9** (Table 3). COSY correlations indicated that the acyl group in **6** was unbranched and the acyl groups in **7** and **8** were *iso*-configured with the presence of independently connected signals terminating in a 3H triplet and a 6H doublet, respectively (Table 2, Fig. S2). COSY correlations for **9** indicated 2-methyl branching by the presence a 3H triplet indirectly connected upfield to a 1H multiplet via a set of diasterotopic protons and a 3H doublet directly connected to the same 1H multiplet (S2). These data are consistent with compounds being identified as pinobanksin-3-butyrate (**6**), pinobanksin-3-isobutyrate (**7**), pinobanksin-3-isopentenoate (**8**), and pinobanksin-3-(2-methyl)butyrate (**9**) (Table 1). The ^{1}H-NMR spectra showed that **6**–**7** were present in a 1:1 ratio, while **8**–**9** were present in a 4:1 ratio (S5-6). Compounds **6**–**8** have been previously reported in propolis from Europe, Iran, or Mexico (Alday et al., 2015; Marcucci, 1995; Mohammadzadeh et al., 2007), but only **6** has been characterized by NMR (Alday et al., 2015). Compound **9** has been previously isolated from Uruguayan propolis and characterized by NMR (Kumazawa et al., 2002).

Compounds **10** ([M-H] = 369.1391 *m/z*) and **11** ([M-H] = 397.1688 *m/z*) displayed fragmentation patterns consistent with six and eight-carbon acyl groups, respectively (Table 3). The presence of a triplet at ~0.9 ppm and the absence of other doublets or singlets in the alkane region of both ^{1}H-NMR spectra suggested that both acyl groups were unbranched (Table 2). Considerable secondary coupling was observed among the methylene protons in these acyl groups, but a combination of COSY and HMBC correlations were used to resolve their connectivity (Fig 2, Fig S2). These data support **10** and **11** being identified as pinobanksin-3-hexanoate (**10**) and pinobanksin-3-octanoate (**11**) (Table 1). To the best of our knowledge, this is the first report and characterization of pinobanksin-3-octanoate (**11**) from any source; however, pinobanksin-3-hexanoate (**10**) has been previously reported in European propolis and identified by MS alone (Marcucci, 1995).

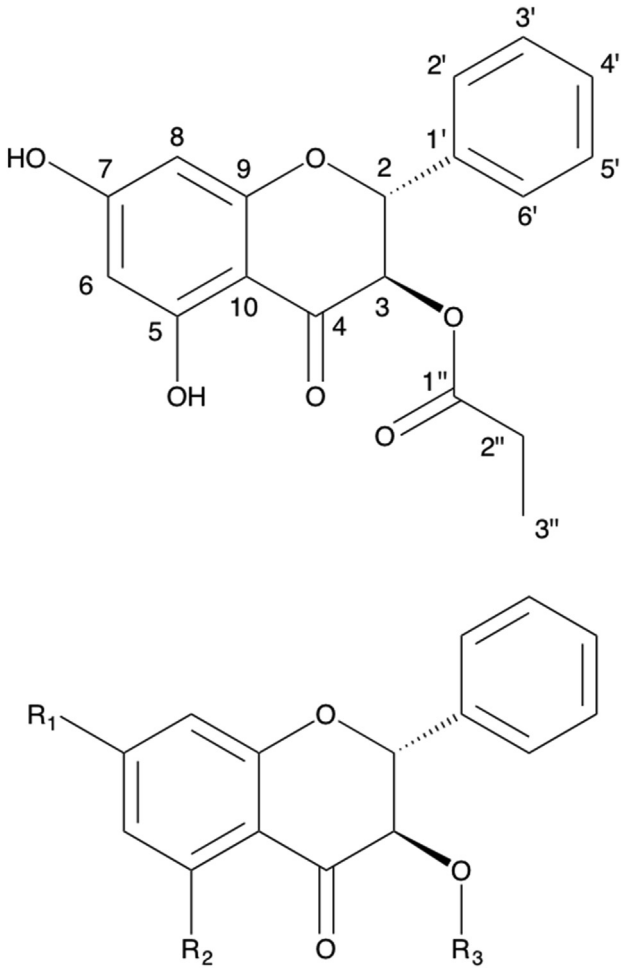


Fig. 1. Generalized flavonoid carbon numbering system.

2.2. Inhibition of P. larvae and A. apis

Pure compounds were screened for preliminary activity against *P. larvae* in broth dilution assays at 50 μg/ml, which is approximately the IC_{50} of the crude NV propolis extract (Wilson et al., 2015). Compounds **1**–**5** were less active than crude extract and were not investigated further (data not shown), while **6**–**11** were substantially more active than crude extract. Since **6**–**7** and **8**–**9** could not be separated, these compounds were tested as mixtures.

Broth dilution assays for compounds **6**–**11** gave IC_{50} values of 23, 14, 8, and 7 μg/ml (68, 39, 22, 17 μM) for compounds **6**–**7**, **8**–**9**, **10**, and **11**, respectively, against *P. larvae* (Fig. 3a, Table 1). In comparison, the IC_{50} of tylosin, the antibiotic used to treat *P. larvae* in the field, was 0.3 μg/ml (0.3 μM) in our assay (Wilson et al., 2015). There appeared to be a positive structure-activity relationship between longer acyl groups and *P. larvae* inhibition (Table 1). Another report demonstrated a parabolic relationship between acyl group carbon number and anti-*S. aureus* activity when flavonoids were synthetically acylated at the 3 position, with the optimum length being 8 to 10 carbons (Stapleton et al., 2004). Re-evaluation of previous LC-MS data (Wilson et al., 2015) established the presence of compounds **6**–**11** in propolis samples from 12 locations across the continental U.S. Interestingly, increased peak areas for compounds **10** and **11** were strongly correlated with lower IC_{50} values in U.S. propolis samples ($R^2 = 0.88$ and 0.79, respectively); however, the correlations between lower IC_{50} and increasing peak areas of compounds **6**–**7** and **8**–**9** were much weaker ($R^2 = 0.51$ and 0.33, respectively). This indicated a connection between the overall anti-*P. larvae* activity of propolis produced in the U.S. and the relative amount of compounds **10** and **11** present in a given sample, regardless of other 3-acyl pinobanksin derivatives present.

Compounds **6**–**11** were also tested for activity against *A. apis* in broth dilution assays, with IC_{50} values of 3, 3, 9, and >100 μg/ml (7.8, 8.3, 23, >250 μM) for compounds **6**–**7**, **8**–**9**, **10**, and **11**, respectively (Fig. 3b, Table 1). Interestingly, the structure-activity relationship observed for *A. apis* was opposite that of the

Table 2
^{1}H and ^{13}C-NMR spectroscopic data. Spectra were obtained in methanol-*d4* and *J* values are reported in Hz.

Position	Pinobanksin-3-propanoate (**5**)		Pinobanksin-3-isobutyrate (**7**)		Pinobanksin-3-isopentanoate (**8**)		Pinobanksin-3-octanoate (**11**)	
	δ_H	δ_c	δ_H	δ_c	δ_H	δ_c	δ_H	δ_c
2	5.38 d ($J = 11.8$)	72.0	5.40 d ($J = 11.9$)	81.0	5.41 d ($J = 12.0$)	81.1	5.41 d ($J = 11.82$)	81.0
3	5.83 d ($J = 11.8$)	80.8	5.84 d ($J = 12.0$)	71.7	5.88 d ($J = 12.0$)	71.8	5.87 d ($J = 11.82$)	72.0
4		194.2		191.2		191.3		191.1
5		166.5		163.5		163.9		163.7
6	5.96 d ($J = 2.1$)	96.3	5.96 d ($J = 2.0$)	96.3	5.97 d ($J = 2.1$)	96.4	5.96 d ($J = 2.05$)	96.6
7		170.3		167.6		167.7		168.2
8	5.95 d ($J = 2.1$)	95.5	5.94 d ($J = 2.0$)	94.8	5.94 d ($J = 2.1$)	95.2	5.94 d ($J = 2.08$)	95.3
9		165.1		162.5		162.5		162.3
10		103.2		100.4		100.4		100.3
1′		138.4		135.3		135.6		135.4
2′	7.50 m	127.3	7.51 m	127.1	7.51 m	127.4	7.52 m	127.3
3′	7.40 m	128.3	7.39 m	128.1	7.40 m	128.2	7.42 m	128.3
4′	7.40 m	128.3	7.39 m	128.1	7.40 m	128.2	7.42 m	128.3
5′	7.40 m	128.3	7.39 m	128.1	7.40 m	128.2	7.42 m	128.3
6′	7.50 m	127.3	7.51 m	127.1	7.51 m	127.4	7.52 m	127.3
1″		175.4		175.3		174.7		171.8
2″	2.25 m	29.2	2.48 m ($J_{app} = 7.0$)	33.3	2.11 m	42.2	2.24 m	33.2
3a″	0.95 t ($J = 7.6$)	10.2	0.89 d ($J = 7.0$)	17.4	1.86 m ($J_{app} = 6.8$)	25.2	1.42 m	24.4
3b″			1.03 d ($J = 7.0$)	17.4				
4a″		12.3			0.75 d ($J = 6.7$)	21.0	1.10 m	28.3
4b″					0.73 d ($J = 6.7$)	21.0		
5″							1.19 m	28.5
6″							1.19 m	31.2
7″							1.30 m	22.2
8″							0.90 t ($J = 7.14$)	12.9

Table 3
Mass spectral data for compounds 5–11. Exact mass was calculated from hypothetical elemental compositions based on NMR and LC-ESI-TOFMS data. LC-MS (35eV) positive ion fragments ≥ 2% of the base-peak are shown with unit mass resolution. Spectra for compounds **6**–**7** and **8**–**9** were identical. Full spectra can be found in the supplementary data.

Compound	Calc. exact mass $[M-H]^-$	Pseudomolecular ion $[M-H]^-$	Fragment (intensity)	Ion Annotation
5	327.0860	327.0872 m/z	272.8 (100)	Partial acyl group loss - C_3H_5O
			254.8 (16)	Full acyl group loss - $C_3H_5O_2$
			226.8 (11)	Loss of CO
			152.8 (2)	Predicted $A^{1,3+}$ ion
6 & **7**	341.1025	341.1072 m/z	272.9 (100)	Partial acyl group loss - C_4H_7O
			254.9 (22)	Full acyl group loss - $C_4H_7O_2$
			226.9 (11)	Loss of CO
			152.8 (2)	Predicted $A^{1,3+}$ ion
8 & **9**	355.1182	355.1182 m/z	272.9 (100)	Partial acyl group loss - C_5H_9O
			254.9 (27)	Full acyl group loss - $C_5H_9O_2$
			226.9 (9)	Loss of CO
			152.8 (4)	Predicted $A^{1,3+}$ ion
10	369.1338	369.1391 m/z	272.9 (100)	Partial acyl group loss - $C_6H_{11}O$
			254.9 (11)	Full acyl group loss - $C_6H_{11}O_2$
			226.8 (4)	Loss of CO
			152.8 (4)	Predicted $A^{1,3+}$ ion
11	397.1651	397.1688 m/z	272.8 (100)	Partial acyl group loss - $C_8H_{15}O$
			254.9 (9)	Full acyl group loss - $C_8H_{15}O_2$
			226.8 (4)	Loss of CO
			152.8 (4)	Predicted $A^{1,3+}$ ion

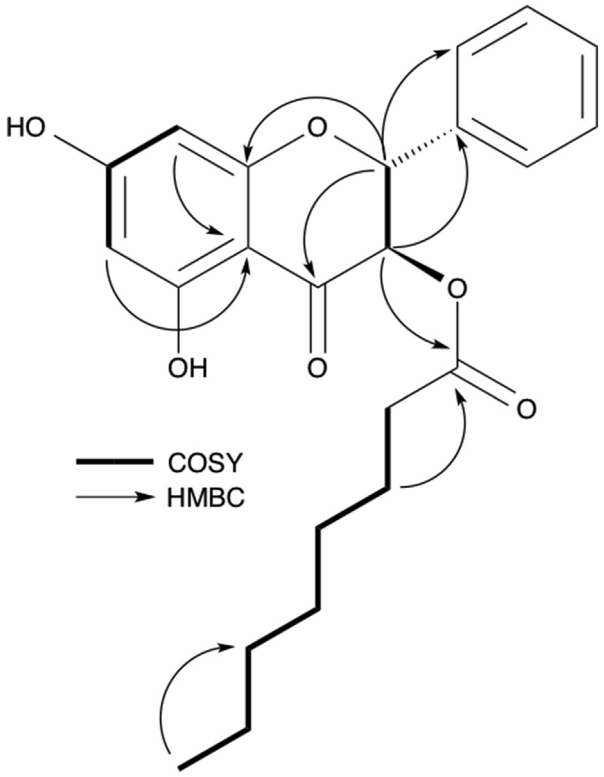


Fig. 2. Key COSY and HMBC correlations for compound **11**.

structure-activity relationship observed for *P. larvae*, with shorter acyl groups corresponding to increased *A. apis* inhibition (Table 1). Since there are no compounds used to treat *A. apis* for practical comparison, the agricultural fungicide benomyl was used as a positive control. The IC_{50} of benomyl was 4 μg/ml (15 μM) in our assay (Wilson et al., 2015), which was higher than the IC_{50} of compounds **6**–**9** (~8 μM) and comparable to the IC_{50} of compound **10** (23 μM). Given their activity and natural source, compounds **6**–**10** might be promising candidates for development as treatments or preventatives of chalkbrood, but more research is needed to understand how resin compounds interact with pathogens in the hive.

It is potentially significant that our isolation strategy produced much higher yields of compounds **6**–**9** than **10**–**11** (70 mg vs. 5.4 mg, S1). This indicates that the compounds most active against *A. apis* were much more abundant than the compounds most active against *P. larvae* in the original propolis sample, and this difference might play a role in the ability of propolis to prevent chalkbrood vs. American foulbrood in the hive. The peak area of compounds **6**–**10** in U.S. propolis samples did not correlate with our previously reported anti-*A. apis* activity (Wilson et al., 2015), suggesting that bioassay-guided fractionation specifically targeting *A. apis* may yield more antifungal compounds.

2.3. Botanical sources of isolated compounds

Resin forager behavior was tracked in the area of Fallon, NV, by previously described methods (Wilson et al., 2013) to determine the botanical source of compounds **6**–**11**. Surface resins were collected from local plants available to bees including *Ericameria nauseosa* (rubber rabbitbrush – two individuals), *Chrysothamnus viscidifloras* (green rabbitbrush – two individuals), *Iva axillaris* (poverty weed – three individuals), *Grindelia squarrosa* (curlycup gumweed – four individuals), unidentified hybrid poplar (*Populus* sp. – one individual), and Fremont poplar (*P. fremontii* – five individuals) by washing resinous leaves or buds in acetonitrile. Resin extracts were readily distinguishable by LC-MS (Fig. 4), though the differences between *P. fremontii* and the hybrid poplar were subtle. Most notably, the hybrid poplar resin extract contained two peaks (RT = 10.9, m/z = 271.1 and RT = 11.2, m/z = 279.2) that were not present in any of the *P. fremontii* resin extracts.

Nine total resin foragers were captured returning to 5 colonies throughout three different apiaries around Fallon, and all bees were released alive at the site of their capture. Resin was removed from the hind legs of foragers with steel pins, extracted in acetonitrile, and compared with plant resin extracts via LC-MS. This analysis confirmed that eight bees carried resin from *P. fremontii* and one bee carried resin from the hybrid poplar (Fig. 4). Compounds **6**–**11** could only be detected in *P. fremontii* or hybrid poplar resin (Fig. 4), supporting the hypothesis that the original propolis sample was

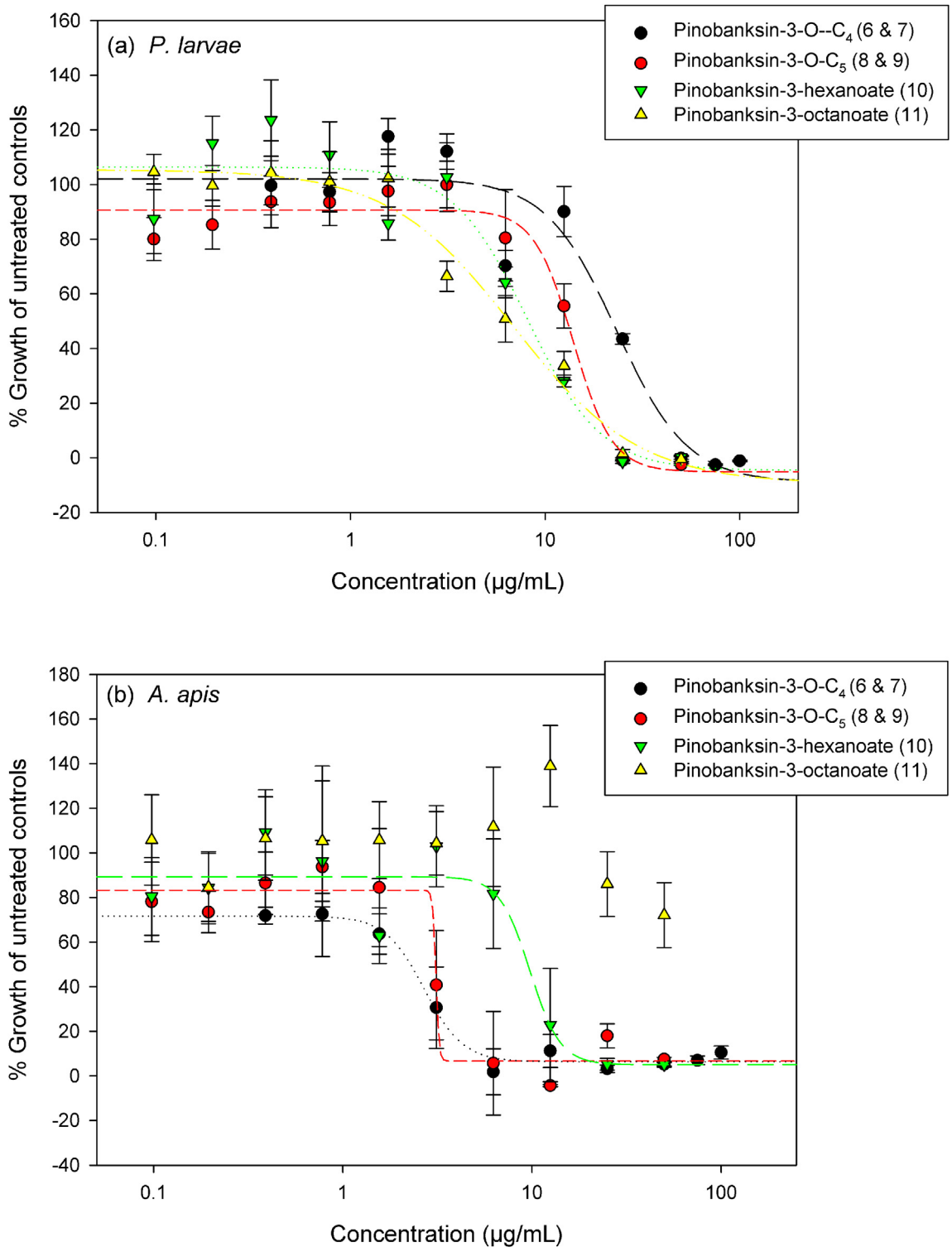


Fig. 3. Dose-dependent inhibition of *P. larvae* (a) and *A. apis* (b). Microbial growth in broth dilution assays was measured as a percent optical density (OD_{600}) relative to untreated controls. Compounds **6**–**7** and **8**–**9** were tested as mixtures, respectively, since they could not be separated on the preparative scale. N = 3 for all treatments.

likely composed of *P. fremontii* and/or hybrid poplar resin. Since hybrid poplars are not common in the Fallon landscape except where intentionally planted for research near one of our resin forager sampling sites (E. Eldridge, personal communication), *P. fremontii* is likely the main botanical source of compounds **6**–**11**.

To manage the environment in order to maximize the antimicrobial benefit of propolis to bees, it is important to know the distribution of active resin compounds among plants in the environment. The presence of compounds **6**–**11** in propolis samples from across the U.S. suggested a common botanical source, even though the collection sites were geographically distant. 3-Acyl dihydroflavonols have been previously reported in *Populus* resins

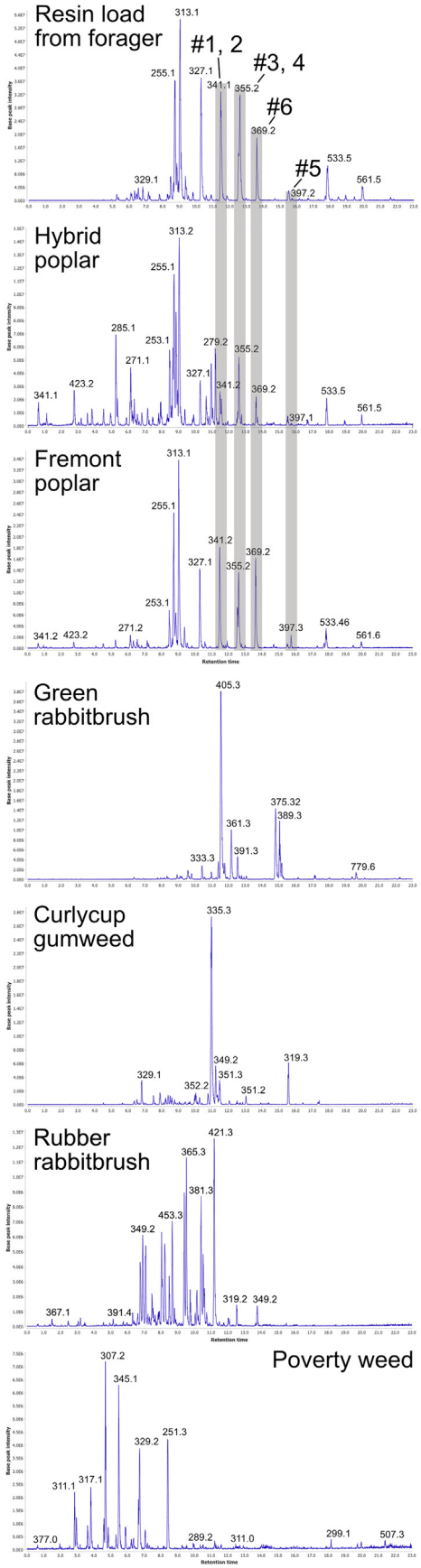


Fig. 4. LC-MS profiles of resins from plants and bees collected around Fallon, NV. Extracts were normalized to 1 mg/ml. The resin profiles of eight bees matched by GC-MS fragmentation analysis (Greenaway and Whatley, 1990; English et al., 1991, 1992) and different *Populus* spp. are widespread and abundant in the U.S. LC-MS analysis of representative North American *Populus* resin extracts from Wilson et al., 2013 indicated that the relative concentrations of compounds **6**–**11** varied among *P. deltoides*, *P. balsamifera*, *P. angustifolia*, *P. nigra*, *P. trichocarpa*, and *P. fremontii* resins (Table 4, Fig. S9). There appeared to be a concentration gradient of the active compounds by species, and it would be interesting to determine how this affects the functional diversity and redundancy of poplar resins in preventing *P. larvae* or *A. apis* infection. Understanding the specific benefit and interchangeability of resinous plants will allow beekeepers to intelligently design or choose environments that promote bees' natural defenses.

3. Conclusions

Bioassay-guided fractionation against *P. larvae* was used to isolate 11 structurally related flavanols from Fallon, NV, propolis. Of these isolated compounds, half were determined to be 3-acyl dihydroflavonols (compounds **4**–**11**) (Table 1). To our knowledge, this is the first structural characterization of compounds **5**, **7**, and **8** by NMR analysis (Table 2, Figs. S4-S7) and the first report of compound **11** (Table 2, Fig. S8).

Compounds **6**–**11** were better inhibitors of *P. larvae* growth than crude NV propolis extract and their IC_{50} values against *P. larvae* and *A. apis* are reported in Table 1. Antimicrobial activity was related to acyl group size where compounds with shorter acyl groups were better inhibitors of *A. apis*, while compounds with longer acyl groups were better inhibitors of *P. larvae* (Table 1). Tylosin displayed much greater anti-*P. larvae* activity compared to compounds **6**–**11**; however, the anti- *A. apis* activity of compounds **6**–**9** against was comparable to the benomyl control. The relative concentrations of compounds **10** and **11** were highly correlated with the anti-*P. larvae* activity observed for U.S. propolis samples in our previous study, indicating that these compounds could be very important over a large geographic area.

Poplar trees (*Populus* spp.) have been regarded as a common source of propolis in the U.S., however this hypothesis is only supported by scattered scientific observations (Crane, 1990; Bankova, 2006; Wilson et al., 2013). Chemical tracking of resin forager behavior indicated that the source of our isolated compounds was most likely *P. fremontii*, however, compounds **6**–**11** were also found in other North American *Populus* resins. These studies support the hypothesis that poplars are a major source of propolis in the U.S. and are perhaps very important plants for honey bee health. Overall, this work connects specific compounds found in *Populus* resins with the antimicrobial benefits that bees derive from propolis deposition.

4. Experimental

4.1. Propolis sampling

Propolis was collected from commercial propolis traps (Mann Lake Ltd., cat. # HD-370) placed on nine managed colonies of *Apis mellifera* in the Fallon, NV, area in 2009. Propolis samples were stored in sealed glass jars at −20 °C. This is the same Fallon, NV, propolis sample used in a previous study (Wilson et al., 2015). A voucher sample is deposited in the Spivak lab propolis collection

P. fremontii, while the resin profile of one bee matched hybrid poplar. The active compounds isolated from Fallon, NV, propolis are highlighted where they occurred in resins loads and *Populus* spp., but were noticeably absent in other resin plants.

Table 4
Relative quantitation of active 3-acyl dihydroflavonols in North American *Populus* resins. Resin extracts from representative greenhouse-grown *Populus* spp. from Wilson et al., 2013 were normalized to 1 mg/ml and re-analyzed for compounds **6**–**11** by co-elution on LC-MS. Percent peak area is normalized to the largest peak for each compound, which was always the peak from *P. nigra*. Extracted ion chromatograms can be found in S9.

Compound(s)	*P. nigra*	*P. fremontii*	*P. angustifolia*	*P. trichocarpa*	*P. deltoides*	*P. balsamifera*
6–**7**	100%	21%	3%	47%	7%	1%
8–**9**	100%	41%	12%	13%	7%	9%
10	100%	60%	47%	34%	20%	11%
11	100%	70%	48%	42%	38%	24%

(#15).

4.2. General experimental procedures

TLC was performed on silica gel 60 (F-254) (Merck, 5719-2) and developed with MeOH:$CHCl_3$ (5:95 v/v) containing 0.2% glacial AcOH. Plates were evaluated by UV-induced fluorescence at 254 nm and 365 nm with a UVGL-25 hand lamp (UVP – Upland, CA) and by sulfuric acid/vanillin staining with heat application (15 g vanillin, 250 ml EtOH, 2.5 ml concentrated H_2SO_4).

The absorbance pattern of pure compounds in MeOH was determined using a Hewlett-Packard 8453 spectrophotometer. UV shift analysis with $AlCl_3$ and NaOAc was performed according to established methods (Markham, 1982). The circular dichroism of pure compounds in MeOH was measured using a JASCO J-815 spectrometer.

Reversed-phase UPLC-MS was used to characterize purified compounds. Fractions were monitored for uniqueness using an Acquity single-quadrupole LC-MS system (Waters – Milford, MA). The accurate mass of purified compounds was measured using a G2 Synapt LC-TOF MS in negative ionization mode (Waters – Milford, MA), while fragmentation analysis was performed with an AmaZon LC-Trap in positive ionization mode (Bruker – Billerica, MA) at 35eV. Although the signal from isolated compounds was better in negative ionization mode, positive ion mode was used for fragmentation analysis to match literature resources. LC conditions were as follows: column = Zorbax Eclipse XDB C_{18} 2.1 × 100 mm 1.8 μm particle size column (Agilent), A = H_2O (0.1% HCO_2H), B = CH_3CN (0.1% HCO_2H), flow rate = 0.35 ml/min. Gradient conditions were as follows: time = 0 min, A = 90%, B = 10%; time = 20 min, A = 10%, B = 90%; time = 22 min, A = 10%, B = 90%; time = 25 min, A = 90%, B = 10%.

^{1}H-NMR, COSY, HMBC, and HMQC experiments for compounds **1**–**4** were performed on Varian VI-400 MHz or VI-500 MHz NMR spectrometers. HMQC, HMBC, and [^{13}C]-NMR experiments for compounds **5**–**11** were performed on a VI-600 MHz NMR or a Bruker Advance 700 MHz NMR. All compounds were analyzed in [$^2H_4$]methanol (Sigma or Cambridge Isotopes).

4.3. Extraction and isolation

Powdered propolis (80 g) was extracted with EtOH:H_2O (1 L, 70:30 v/v) at room temperature over two days, with 40 min/day of sonication. This extraction was followed by two additional extractions with EtOH:H_2O (0.5 L x 2, 70:30 v/v) at room temperature using the same procedure. These ethanolic extracts were pooled, concentrated in a rotary evaporator, and taken up in MeOH (Sigma, Chromosolv Plus grade). The pooled extract was diluted to MeOH:H_2O (10:90 v/v) and partitioned successively against two equal volumes of hexanes over 24 h periods (Fisher Scientific, HPLC grade). The remaining MeOH:H_2O layer was then partitioned successively against two equal volumes of CH_2Cl_2 over 24 h periods (Sigma, Chromosolv grade).

The Reveleris flash chromatography system (Grace, Deerfield, IL) was used to perform normal-phase separations on 40 g silica cartridges (Grace). The CH_2Cl_2 fraction residue (10 g) was fractionated using a $CHCl_3$:MeOH gradient (A = $CHCl_3$, B = MeoH, flow rate = 25 ml/min. Gradient conditions were as follows: time = 0, A = 100%, B = 0%; time = 5 min, A = 90%, B = 10%, time = 10.5 min, A = 88%, B = 12%; time = 15.5 min, A = 0%, B = 100%, time = 18 min, A = 0%, B = 100%). Evaporative light-scattering detection (ELSD) was used to detect 13 unique fractions (5.2–820.7 mg), with a total recovery of 2.14 g.

Three flash chromatography fractions with enriched activity compared to crude extract were combined based on chemical similarity and further separated on a Sephadex LH-20 (170 g, GE Healthcare Life Sciences – Pittsburg, PA) open column (3 cm × 33.75 cm) using MeOH as the elution solvent. Fractions were collected in 20 ml volumes, with a total recovery of 1.7 g. Fractions #3–5 (245.1, 412.4, and 259.4 mg) showed enriched activity compared to crude extracts.

Pure compounds were isolated from active Sephadex LH-20 fractions using reversed-phase Prep-HPLC (Agilent 1200 Series Preparatory HPLC system, Agilent Technologies – Santa Clara, CA) on a Zorbax Eclipse XDB C_{18} PrepHT 21.2 × 250 mm, 7 μm particle size column (Agilent) with a H_2O:MeOH gradient (A = H_2O, B = MeOH, flow rate = 20 ml/min. Gradient conditions where as follows: time = 0 min, A = 60%, B = 40%; time = 20 min, A = 5%, B = 95%; time = 25 min, A = 5%, B = 95%). Eluting compounds were monitored for absorbance at 254 nm and 320 nm. This separation yielded four known compounds from Sephadex fraction #3 (**6**–**7**, 24.5 mg; **8**–**9**, 44.5 mg), five known compounds from Sephadex fraction #4 (**2**, 0.8 mg; **3**, 2 mg; **4**, 72.2 mg; **5**, 44 mg; **10**, 1.8 mg), and one known compound from Sephadex fraction #5 (**1**, 3.5 mg) (Table 1, Fig. S1). One unknown compound, pinobanksin-3-octanoate (**11**, 3.6 mg), was isolated from Sephadex fraction #3 (Table 1, Fig. S1).

4.3.1. Pinobanksin-3-octanoate

Orange oil, 3.6 mg; UV (MeOH) λ_{max} (log ε) 295 (3.09), 335 (2.64) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$) = 324 (0.32), 287 (−1.05) nm; For ^{1}H and ^{13}C NMR spectroscopic data see Table 2; ESI-TOF-MS m/z = 397.1688 $[M-H]^-$ (calculated for $C_{23}H_{26}O_6$, 397.1651); For MS-MS data see Table 3.

4.4. Antimicrobial assays

Fractionation of NV propolis was guided by broth dilution assays against planktonic ERIC type I *P. larvae* (NRRL #B-2605, ATCC 9545, LMG 9820) as previously described (Wilson et al., 2015). Briefly, compounds in MeOH were dried to residue in microplate wells with N_2 gas and then wells were inoculated with a 1/100 dilution of overnight *P. larvae* culture in brain/heart infusion broth. The optical density (OD) of cultures was measured at 600 nm after 6 h of shaking and incubation at 37 °C, with the optical density of each well at 0 h subtracted as background. OD_{600} values were normalized as the percent growth of untreated controls. Assays were performed in triplicate.

Broth dilution assays against spores from mated *A. apis* reference strains (USDA #7405 and USDA #7406) and planktonic *P. larvae* (NRRL B-2605) were performed as previously described (Wilson et al., 2015). Briefly, compounds in MeOH were dried to residues in microplate wells with N_2 gas and then inoculated with 1.98×10^6 *A. apis* spores in MY-20 broth or a 1:100 dilution of overnight *P. larvae* culture in BHI broth supplemented with 1 mg/L thiamine HCl. The OD_{600} of cultures was evaluated after 65 h of shaking and incubation at 31 °C for *A. apis* and after 6 h of shaking and incubation at 37 °C for *P. larvae*. Assays were performed in triplicate. The optical density of each well at 0 h was subtracted as background, and OD_{600} was normalized as the percent growth of untreated controls. IC_{50} values were calculated by fitting a four-parameter logistic curve to the sigmoidal inhibition data as previously described (Wilson et al., 2015). R^2 values for data fit in Fig. 3a were 0.94 for compounds **6**–**7**, 0.97 for compounds **8**–**9**, 0.94 for compound **10**, and 0.98 for compound **11**. R^2 values for data fit in Fig. 3b were 0.99 for compounds **6**–**7**, 0.96 for compounds **8**–**9**, and 0.90 for compound **10**.

4.5. Determination of botanical sources and prevalence of compounds in Populus spp. resins

In July 2014, nine resin foragers were captured from full sized colonies in three different apiaries near Fallon, NV by blocking hive entrances for 15 min and capturing returning resin foragers by hand in wire mesh cages. Foragers were chilled on ice and resin loads were removed with insect pins. For comparison, surface resins were collected from the leaves or buds of *Ericameria nauseosa* (rubber rabbitbrush, Asteraceae), *Chrysothamnus viscidifloras* (green rabbitbrush, Asteraceae), *Iva axillaris* (poverty weed, Asteraceae), *Grindelia squarrosa* (curlycup gumweed, Asteraceae), *P. fremontii* (Fremont poplar, Salicaceae), and an unknown hybrid poplar (*Populus* sp., Salicaceae) by washing whole plant material in CH_3CN for 15 min. Plant material was authenticated by Dr. Eric Eldrige from the USDA-NRCS research station in Fallon, NV, and voucher species were deposited in the Cohen lab collection. LC-MS analysis was performed with the Acquity system described above.

The presence of compounds **6**–**11** was assessed in resin extracts from representative samples of North American *Populus* spp. with the Acquity LC-MS system described above. It was previously found that resins from different *Populus* spp. are compositionally distinct, yet varied little among individuals of the same species when grown under uniform greenhouse conditions (Wilson et al., 2013). Peak areas of extracted ion chromatograms were calculated using MassLynx software (Waters – Milford, MA).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2017.02.020.

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